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(71) Applicant (for all designated States except US): BIO-SYNTECH LTD. [CA/CA]; P.O. Box 125, Succursale St-Martin, Laval, Québec H7V 3P4 (CA).			
(72) Inventors; and (75) Inventors/Applicants (for US only): CHENITE, Abdelatif [CA/CA]; 28, rue Béthune, Kirkland, Québec H9H 4H6 (CA). CHAPUT, Cyril [FR/CA]; 3333-44 Edouard-Montpetit, Montréal, Québec H3T 1K4 (CA). COMBES, Christèle [FR/CA]; Appartement 6, 32, avenue de Suisse, F-31250 Ramonville St-Agne (FR). JALAL, Fayrouz [CA/CA]; 309-4850 Côte des Neiges, Montréal, Québec H3V 1G7 (CA). SELMANI, Amine [CA/CA]; 2084 Jessop, Laval, Québec H7S 1X1 (CA).			
(74) Agent: COTE, France; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).			
(54) Title: TEMPERATURE-CONTROLLED pH-DEPENDANT FORMATION OF IONIC POLYSACCHARIDE GELS			
(57) Abstract			
<p>The present invention relates to a temperature-controlled pH-dependant formation of ionic polysaccharide gels, such as chitosan/organo-phosphate aqueous systems, and methods of preparation thereof. While chitosan aqueous solutions are pH-dependant gelating systems, the addition of a mono-phosphate dibasic salt of polyol or sugar to chitosan aqueous solutions leads to further temperature-controlled pH-dependant gelation. Solid organo-phosphate salts (1-20 % w/v) are added and dissolved at low temperature (10 °C) within 0.5 to 4.0 % w/v chitosan in aqueous acidic solutions. Aqueous chitosan/organo-phosphate solutions are initially stored at low temperatures (4 °C), then endothermally gelated within the temperature range of 30 to 60 °C. Chitosan/organo-phosphate solutions rapidly turn into gels at the desired gelation temperature. Gelation can be <i>ex vivo</i> within any receivers or molds, or <i>in situ</i> in animals or humans (<i>in vivo</i>) so as to fill a tissue defect or cavity.</p>			

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**TEMPERATURE-CONTROLLED PH-DEPENDANT  
FORMATION OF IONIC POLYSACCHARIDE GELS**

**BACKGROUND OF THE INVENTION**

5    (a) Field of the Invention

The present invention relates to a temperature-controlled pH-dependant formation of ionic polysaccharide gels, such as chitosan/organo-phosphate aqueous systems, and methods of preparation thereof.

10   (b) Description of Prior Art

Chitosan is a commercially available inexpensive polymer, a derivative of chitin or poly(N-acetyl-glucosamine) materials. Chitosan is mainly composed of D-glucosamine units that are generated through catalyzed  
15 N-deacetylation of chitin, an insoluble biopolymer extracted from hard shells of marine living animals (fishes, crustaceans, shrimps, crabs...) or synthesized by natural organisms (zygomycete, fungi...). Chitosan is expected to have good viscoelastic properties, and  
20 has adequate tissue compatibility and biodegradability which renders it ideal for bioactive and resorbable implants. Poly-D-glucosamine chains are also known to potentially attach a large number of proteoglycan molecules and coexist with fibrous collagens to form aqueous  
25 gels. It is believed that the role of proteoglycans within the gel is to retain water and supply appropriate viscoelasticity. Resulting extracellular matrices are expected to offer compatible environments for cellular proliferation and tissue formation, especially  
30 for skin, ligament, bone and cartilage cells. As a consequence, chitosan attracts great interest for scaffolding or supporting materials of bioengineered artificial tissues.

Moreover, chitin and partially-acetylated chitosan derivatives have been extensively investigated for  
35 therapeutic substances or implantable materials. Bio-

compatibility of chitosan-based materials has been evaluated specifically for blood, wounds and bone. Immunological and genotoxic activities as well as stimulatory effects on macrophagic action have been also studied with various chitosan materials.

Chitosan and its derivatives has been widely explored for drug delivery system through gels (Ohya Y. et al. (1993) *J. Micro-encapsulation*, 10(1):1-9). Peptides delivery with chitosan was proposed to be effected nasally. Cationic colloidal drug carriers were proposed from chitosan-polycaprolactone systems. Wound healing and reconstructive devices made of chitosan materials have been proposed for open or corneal wounds, periodontal tissues and skin. Chitosan was specially evaluated in bone and dura matter and as an hemostatic.

Entrapment of living biologicals (cells, enzymes, etc...) have been investigated with different chitosan products, however, in nearly all cases, living cells have been encapsulated within alginate/chitosan microbeads. Encapsulation of chondrocytes (cartilage cells) were proposed within calcium-alginate/chitosan beads.

Gelation of chitosan through polyphosphates has been promoted for encapsulating cells such as neural or musculo-skeletal tissues. Generally, chitosan in an acid/water medium was loaded with cell suspensions, and the resulting mixture was dropped in a buffered pentasodium tri-polyphosphates so as to form cell-loaded chitosan beads and capsules. Entrapment of neural cells within polyphosphate-gelated chitosan beads has led to good cellular viability but low proliferation rate (Zielinski B.A. et al. (1994) *Biomaterials*, 15(13):1049-1056). No large or specific three-dimensional shaped materials were proposed (Zielinski B.A.

et al. (1994) *Biomaterials*, 15(13):1049-1056). Poly-saccharide capsules have been proposed for entrapping physiologically active cells such as Langerhans Islets (U.S. Patent No. 4,391,909). Chitosan/hydrochloride cisplatin mixture were cross-linked and proposed as drug delivery systems.

Chitosan derivatives have been incorporated in numerous carrier composition or drug formulation. Chitosan materials such as wound filling materials or contraceptive products were also proposed (U.S. Patents Nos. 4,956,350 and 4,474,769). Chitosan gels were again reported as supports for immobilizing and encapsulating living biomaterials such as cells, bacteria and fungi (U.S. Patent No. 4,647,536). Ophthalmic drug delivery systems made of chitosan were also proposed for *in situ* gelating and forming (U.S. Patent No. 5,422,116).

In U.S. Patent No. 4,659,700, chitosan gels were prepared from glycerol/acid/water systems as biodegradable carriers for drug delivery. The resulting chitosan gels were reported to remain quite stable, keeping intact their three-dimensional shape for long periods and over a wide range of temperatures, particularly between 4 and 40°C. Gels and gel-like materials were processed by dissolving 1.0 to 4.0% w/v chitosan within acid-water-glycerol solutions wherein acetic, formic or propionic acid and 10-90% glycerol proportions are used preferentially, and by neutralizing with liquid bases such the sodium, ammonium and potassium hydroxides or ammonia vapors. The pH of the resulting chitosan-glycerol gel materials is about pH 7.0. After neutralization, the resultant mixtures turn into gels upon standing, such gels resulting apparently from the interaction of chitosan, glycerol and water. No free glycerol or water were reported as being apparent. It must be noted, however, that such three-dimensionally shaped

chitosan-glycerol gels will occur only when the solution is previously neutralized with a base. One-piece three-dimensional gels can be molded easily as well as gel-like membranes. The role of the glycerol component and chitosan-glycerol interactions is not elucidated.

*In situ* formed gels were also proposed with ionic polysaccharides in U.S. Patent No. 5,587,175. A composition can be used as a medical device for drug delivery, the application of a diagnostic agent, or the prevention of post-operative adhesions, and is composed of an aqueous liquid vehicle which is capable of being gelled *in situ*. It includes at least one ionic polysaccharide, at least one film forming polymer, and a medicament or pharmaceutical agent, water, and optionally, a counter-ion capable of gelating the ionic polysaccharide (U.S. Patent No. 5,587,175). However, the gelation is reached by interaction between the ionic polysaccharide and the film-forming polymer, or by counter-ion induced cross-linking of the ionic polysaccharide. Other *in situ* forming gels are based upon polyoxyalkylene composition (U.S. Patent No. 4,185,618) or polyoxyalkylene/polysaccharide mixture (U.S. Patent No. 5,126,141) or alginate/cation mixture *in situ* (U.S. Patents Nos. 4,185,618 and 5,266,326).

It would be highly desirable to be provided with a temperature-controlled pH-dependant formed polysaccharide gel which could be used to encapsulate cells and cellular material while retaining their biological activity.

It would be highly desirable to be provided with such a polysaccharide gel which would retain its solid or gel state at the physiological temperature or 37°C.

### SUMMARY OF THE INVENTION

One aim of the present invention is to provide a temperature-controlled pH-dependant formed polysaccharide gel which could be used to encapsulate cells and cellular material while retaining their biological activity.

Another aim of the present invention is to provide a polysaccharide gel which would retain its solid or gel state at the physiological temperature or 37°C.

Another aim of the present invention is to provide a method for the preparation of such a polysaccharide gel.

In accordance with the present invention there is provided a polysaccharide based gel which comprises:

- a) 0.1 to 5.0% by weight of chitosan or a chitosan derivative; and
- b) 1.0 to 20% by weight of a salt of polyol or sugar selected from the group consisting of mono-phosphate dibasic salt, mono-sulfate salt and a mono-carboxylic acid salt of polyol or sugar;

wherein said gel is induced and stable within a temperature range from 20 to 70°C and is adapted to be formed and/or gelled *in situ* within a tissue, organ or cavities of an animal or a human.

The salt may be any of the following or in any of the following combination:

- a) a mono-phosphate dibasic salt selected from the group consisting of glycerol, comprising glycerol-2-phosphate, sn-glycerol 3-phosphate and L-glycerol-3-phosphate salts;
- b) a mono-phosphate dibasic salt and said polyol is selected from the group consisting of histidinol, acetol, diethylstilbestrol, indole-glycerol, sorbitol, ribitol, xylitol, arabinitol,

erythritol, inositol, mannitol, glucitol and a mixture thereof;

- 5 c) a mono-phosphate dibasic salt and said sugar is selected from the group consisting of fructose, galactose, ribose, glucose, xylose, rhamnulose, sorbose, erythrulose, deoxy-ribose, ketose, man-  
10 nose, arabinose, fuculose, fructopyranose, keto-glucose, sedoheptulose, trehalose, tagatose, sucrose, allose, threose, xylulose, hexose, methylthio-ribose, methylthio-deoxy-ribulose, and a mixture thereof;
- 15 d) a mono-phosphate dibasic salt and said polyol is selected from the group consisting of palmitoyl-glycerol, linoleoyl-glycerol, oleoyl-glycerol, arachidonoyl-glycerol, and a mixture thereof; and
- 20 e) glycerophosphate salt is selected from the group consisting of glycerophosphate disodium, glycerophosphate dipotassium, glycerophosphate calcium, glycerophosphate barium and glycerophosphate strontium.

A preferred gel in accordance with one embodiment of the present invention is selected from the group consisting of chitosan- $\beta$ -glycerophosphate, chito-  
25 san- $\alpha$ -glycerophosphate, chitosan-glucose-1-glycerophosphate, and chitosan-fructose-6-glycerophosphate.

The solid particulate or water-soluble additives may be incorporated within said polysaccharide gel prior to the gelation.

30 The drugs, polypeptides or non-living pharmaceutical agents may be incorporated within said polysaccharide gel prior to the gelation.

The living microorganisms, plant cells, animal cells or human cells may be encapsulated within said  
35 polysaccharide gel prior to the gelation.



The gel may be formed *in situ* sub-cutaneously, intra-peritoneally, intra-muscularly or within biological connective tissues, bone defects, fractures, articular cavities, body conduits or cavities, eye cul-  
5 de-sac, or solid tumors.

The gel of the present invention may be used as a carrier for delivering pharmaceutical agents *in situ*.

In accordance with the present invention there is also provided a method for producing a polysaccha-  
10 ride gel solution of the present invention, which comprises the steps of:

a) dissolving a chitosan or a chitosan derivative within an aqueous acidic solution of a pH from about 2.0 to about 5.0 to obtain an aqueous  
15 polysaccharide composition having a concentration of 0.1 to 5.0% by weight of a chitosan or of a chitosan derivative;

b) dissolving 1.0 to 20% by weight of a salt of polyol or sugar, wherein said salt is selected  
20 from the group consisting of mono-phosphate dibasic salt, mono-sulfate salt and a mono-carboxylic acid salt, in said aqueous polysaccharide composition of step a) to obtain a polysaccharide gel solution, wherein said polysaccha-  
25 ride gel has a concentration of 0.1 to 5.0% by weight of a chitosan or a chitosan derivative, and a concentration of 1.0 to 20% by weight of a salt of a polyol or sugar, and has a pH from about 6.4 to about 7.4.

30 This method may further comprises a step c) after step b),

c) heating said polysaccharide gel solution at a solidifying temperature ranging from about 20°C to about 80°C until formation of a polysaccha-  
35 ride gel.

A pharmaceutical agent may be added to the polysaccharide gel solution of step b).

The method may further comprises a step i) after step b),

- 5        i) dispensing for gelation the polysaccharide gel solution into a desired receiver, either in a mold or within a tissue, organ or body cavity.

10        The aqueous acidic solution may be prepared from organic or inorganic acids selected from the group consisting of acetic acid, ascorbic acid, salicylic acid, phosphoric acid, hydrochloric acid, propionic acid, formic acid, and a mixture thereof.

15        The polysaccharide gel solution may be kept in a stable ungelled liquid form at a temperature ranging from about 0°C to about 20°C.

      The solidifying temperature is ranging from about 37°C to about 60°C, preferably about 37°C.

      The molecular weight of chitosan is ranging from about 10,000 to 2,000,000.

20        The polysaccharide gel is thermoirreversible or thermoreversible by adjusting the polysaccharide gel pH, when the pH of said polysaccharide gel solution is >6.9, or when the pH of said polysaccharide gel solution is <6.9.

25        The solid particulate additives may be added to the polysaccharide gel solution of step b).

30        The polysaccharide gel solution may be introduced within an animal or human body by injection or endoscopic administration, and gelled *in situ* at a temperature of about 37°C.

      In accordance with the present invention there is also provided the use of the polysaccharide gel for producing biocompatible degradable materials used in cosmetics, pharmacology, medicine and/or surgery.

The gel may be incorporated as a whole, or as a component, into implantable devices or implants for repair, reconstruction and/or replacement of tissues and/or organs, either in animals or humans.

5       The gel may be used as a whole, or as a component of, implantable, transdermal or dermatological drug delivery systems.

10       The gel may be used as a whole, or as a component of, ophthalmological implants or drug delivery systems.

The gel may be used for producing cells-loaded artificial matrices that are applied to the engineering and culture of bioengineered hybrid materials and tissue equivalents.

15       The loaded cells may be selected from the group consisting of chondrocytes (articular cartilage), fibrochondrocytes (meniscus), ligament fibroblasts (ligament), skin fibroblasts (skin), tenocytes (tendons), myofibroblasts (muscle), mesenchymal stem  
20 cells and keratinocytes (skin).

The cells-loaded gel and derived products are devoted to the culture and engineering of artificial articular cartilage and cartilaginous tissues and organs, either for surgical or laboratory testing  
25 applications.

The cells-loaded gel and derived products are devoted to the processing and engineering of living artificial substitutes for ligaments, tendons, skin, bone muscles and any metabolic organs, either for surgical or laboratory testing applications.  
30

The cells-loaded gel and derived products are applied as living substitutes for the replacement of articular cartilages, fibrocartilages, cartilaginous organs, ligaments, tendons, bone tissues or skin.

The cells-loaded hydrogel is gelated *in situ* to induce an ectopic formation of fibrocartilage-like or cartilage-like tissues.

In accordance with the present invention there is also provided the use of loaded polysaccharide gel as injectable or implantable gel biomaterials which act as supports, carriers, reconstructive devices or substitutes for the formation *in situ* of bone-like, fibrocartilage-like or cartilage-like tissues at a physiological location of an animal or a human.

The polysaccharide gel solution may be used for producing a derived gel or hydrogel by 1) incorporating and dissolving at least one complementary polymer within said polysaccharide gel solution, and 2) by allowing said polysaccharide and complementary polymer to interact for a sufficient period of time to turn into a clear three-dimensional gel within a temperature range between 20°C to 60°C.

The complementary polymer is a non-ionic water-soluble polysaccharide or a hydroxyalkyl cellulose.

For the purpose of the present invention the following terms and expressions are defined below.

The term "polysaccharide gel solution" is intended to mean a polysaccharide solution in a stable ungelled liquid form at a temperature ranging from about 0°C to about 15°C which can be gelated or changed to a gel state when heated at the gelating temperature.

The term "gelating temperature" is intended to mean any temperature ranging from about 20°C to about 80°C, preferably between 37°C. to about 60°C, and more preferably at about the physiological temperature or 37°C.

The expression "salts of polyols or sugars" is intended to mean mono-phosphate di-basic salts, mono-sulfate salts and mono-carboxylic acid salts of polyols

or sugars.

The present invention include method of forming different gelated materials, those materials being either molded (customized shapes, tubes, membranes, 5 films...) or formed *in situ* within biological environments (filling of tissue defects).

In a preferred embodiment, the chitosan/organo-phosphate aqueous solution has a pH above the pKa of chitosan and turn into solid gel upon thermal stimulation. This polysaccharide gel can be used as a carrier 10 for drugs or as a non-living therapeutics delivery systems, as substituting materials for tissues and organs and as encapsulants for living cells or microorganisms. Chitosan/organo-phosphate gel matrices are rapidly 15 formed at temperatures between 30 to 60°C. Chitosan/organo-phosphate aqueous systems are used as injectable filling materials, injected and gelated *in situ* for filling and repairing tissue defects.

Glycerol-2-phosphate, glycerol-3-phosphate and 20 glucose-1-phosphate based salts are the preferred disclosed salts in accordance with the present invention.

Chitosan/polyol- or sugar-phosphate and chitosan/polyol- or sugar-sulfate gels can be applied to surgical reconstructive and regeneration uses and drug 25 delivery purposes. They provide thermally reversible or irreversible bioerodible polymeric gels with biologically well-known and compatible components for a broad range of medical/biotechnological applications.

### 30 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the occurrence of thermally-controlled Sol to Gel transition (gel formation) dependent upon the pH of the chitosan/glycerophosphate aqueous solution characterized by the chitosan concentration (% by weight), the glycerophosphate concentra- 35

tion (% by weight) and the pH of the chitosan/glycerophosphate aqueous solution;

Fig. 2 illustrates the Sol/Gel diagram for the chitosan/glycerophosphate systems presented as a relationship between the molar content in glycerophosphate and the ratio of the molar content of glycerophosphate by the molar content of glucosamine units;

Fig. 3 illustrates the Sol to Gel transition (Gel formation) induced by heat and followed versus the temperature by rheologic measurement of the modulus and viscosity parameters;

Fig. 4 illustrates the microstructures of chitosan/glycerophosphate gels formed *in vitro* viewed as observed by Scanning Electron Microscopy on gel samples that have been freeze-dried at -30°C and during 8 hours; and

Fig. 5 illustrates chitosan/glycerophosphate solutions which are injected in rabbits, subcutaneously and intraarticularly in legs or subcutaneously at the torso, and allowed to gelate *in situ* at the body temperature.

#### DETAILED DESCRIPTION OF THE INVENTION

Chitosan is dissolved in acidic aqueous solutions so as to obtain clear aqueous chitosan solutions having pH levels within the range 4.3 to 5.6. The chitosan solutions can be sterilized through filtering or steam-autoclaving, and stored at low positive temperature (4°C). The organo-phosphate component is added to the chitosan solution, preferably at low positive temperature (4°C), then the aqueous chitosan/organo-phosphate mixture is gelated thermally, through an endothermal mechanism, within the temperature range from 30 to 60°C. Once formed the resulting chitosan/organo-phosphate gels are thermally stable upon heating even up to

180°C (in autoclave), particularly in cell culture medium. Bioencapsulation within chitosan/organo-phosphate gels is obtained by incorporating the living cells within the ungelated aqueous chitosan/organo-phosphate solution at a low temperature (4°C). Then the temperature of the resulting mixture chitosan/organo-phosphate/cells is raised to and maintained at 37°C where the gelation occurs in ~1 hour. organo-sulfates or mono-carboxylic acid salt of polyols or sugars play a similar role than organo-phosphates.

chitosan and its derivatives are relatively inexpensive and commercially available materials and represent an attractive group of biocompatible and degradable polymers. They have solid or solution properties which can be modified by changing their chemical composition and/or physico-chemical characteristics. The deacetylation degree and molecular weight have been shown to greatly influence the solution properties, enzymatic degradability and biological activity. Chemical modifications, for instance, have been proposed to neutralize or modify chitosan chains by incorporating carboxylic acid, acetate, glutamic acid, carboxymethyl or sulfate groups. Chemical cross-linking (anhydride, glutaraldehyde, glutamate succinimide-PEG...) of chitosan macromolecules induces covalent bonds to create branched or grafted networks.

Physical gelation of chitosan and its derivatives can be obtained through different techniques:

- a) neutralization (NaOH, KOH, NH<sub>4</sub>OH...) which induces hydrogen bonding between chitosan chains;
- b) ionic complexation with divalent anions (borate, molybdate, polyphosphate, sulfate salts and sulphated macromolecules....) which induces pure electrostatic interactions;
- c) complexation with anionic surfactants (sodium alkyl

sulfate...) which induces electrostatic interactions and surfactant-surfactant hydrophobic interactions.

In accordance with the present invention there is proposed a new gelation mechanism that combines  
5 hydrogen bonding, electrostatic interactions and chitosan-chitosan hydrophobic interactions. It can only be achieved through complex interactions between chitosan macromolecules, water molecules and mono-phosphate dibasic salts of polyols or sugars.

10 Polyols are frequently added to compositions for improving gel properties. Sorbitol and mannitol are currently used as tonicity enhancing agents. Glycerol and polyethylene glycol are proposed as plasticizers. Polyols (-ol: glycerol, sorbitol...) and sugars (-ose:  
15 fructose, glucose, galactose...) were used as thermal stabilizing agents for proteins in solutions (Back J.F. et al. (1979) *Biochemistry*, 18(23):5191-5196). Depending on the selected molecules, they were found to make or break structuring of water, create hydrogen bonding,  
20 electrostatic or hydrophobic interacting, and present endothermic transitions (Back J.F. et al. (1979) *Biochemistry*, 18(23):5191-5196). Polyols and sugars stabilize proteins to heat denaturation through their structuring effect on water and the strengthen of hydrophobic  
25 interactions.

Beta-glycerophosphate disodium or calcium salt, or glycerol-2-phosphate disodium or calcium salt, is a well studied molecule in biological sciences. It is considered as a substrate for alkaline phosphatase  
30 (AL). glycerophosphate is widely used as a cell culture medium supplement for culturing cells isolated from musculo-skeletal tissues, and has been shown to induce or maintain the synthesis of specific matrix components when delivered to bone/cartilage cells in culture  
35 (Chung C.-H. et al. (1992) *Calcif. Tissue Int.*, 51:305-



311; Bellows C.G. et al. (1992) *Bone and Mineral*, 17:15-29). Gelation of chitosan will occur with any grade or purity glycerophosphate while encapsulation of living biologicals would require cell culture tested glycerophosphate. Alpha-glycerophosphate disodium or calcium salt, or glycerol-3-phosphate disodium or calcium salt, is also an organic salt of biological importance (Chung C.-H. et al. (1992) *Calcif. Tissue Int.*, 51:305-311). Glycerophosphate salts are precipitated from glycerophosphoric acids which are obtained through the hydrolysis of lecithin, a well-know biological molecule and phosphatides of eggs, soybean and fishes. Glycerophosphoric acids are present under two isomeric structures, the alpha and beta, wherein the beta-glycerophosphoric acid is optically inactive and the alpha-glycerophosphoric acid is optically active. Glycerophosphoric acid is physiologically active compound, being involves in the catabolism of carbohydrates. Glycerophosphate dehydrogenase was also found active in nerve tissues while glycerophosphate was reported to accelerate the rate of decolonization of methylene blue by guinea pig nerves. Alpha-glycerophosphate interacts with pyruvic acid through oxidation-reduction reactions for producing lactic acid in fresh muscle extracts. Glycerophosphoric acid is currently available under disodium, calcium, magnesium, dipotassium, strontium and barium salts, having a relatively strong basic character. Both alpha- and beta-glycerophosphate salts are inexpensive readily available sources of organic mono-phosphate dibasic salts among the polyol or sugar phosphate salts.

Solubilization of chitosan in aqueous solutions requires the protonation of the amine groups of the chitosan chains which is reached within acidic aqueous solutions having a pH ranging from 3.0 to 5.0. When

solubilized, chitosan remains soluble until a pH about 6.2. Neutralization of acidic chitosan solutions by alkali results in a pH increase as well as a de-protonation of the amine groups. Neutralization of acidic chitosan solutions to a pH above the pKa of chitosan at about 6.3-6.4 results in OH-HN and O-HN interchains and water-chitosan hydrogen bonds which induce a hydrated three-dimensional network, a chitosan gel. At pH above 6.3-6.4, chitosan solutions result systematically into chitosan gels at a normal temperature range (0-60°C). However, admixing of an organo-phosphate to a chitosan aqueous solutions increases the pH of the chitosan/organo-phosphate solutions which remain ungelled and liquid for long periods of time even at pH above 6.5, and up to 7.2. This neo-neutral chitosan/organo-phosphate aqueous solutions (pH 6.5-7.2) will gelate when stimulated by an adequate temperature. The time of gelation is controlled by the temperature. For example, a chitosan/organo-phosphate solution which gelates in about 30 minutes at 37°C, needs only about 2 minutes at 60°C to form a gel.

The mechanism of gelation as well as the gel characteristics have been expected to be similar for all chitosan/organo-phosphate systems. Thus, the gelation of chitosan/ $\beta$ -glycerophosphate solutions which has been investigated in more details can be considered as typical example. The results indicating the pH-dependence and the temperature-dependence of the gelation for chitosan/ $\beta$ -glycerophosphate solutions are summarized in the sol-gel diagrams shown in Figs. 1 and 2. In addition to the gel strength, the rheological experiments represented in Fig. 3, show unambiguously that the gelation of chitosan/ $\beta$ -glycerophosphate solutions occur upon heating. The changes in modulus which appear at about 60°C are symptomatic of the Sol to Gel transition

and Gel formation. This temperature for gel formation will be dependent upon the solution characteristics and heating rate (energy of activation required).

5 The porous structure of chitosan/ $\beta$ -glycerophosphate gels has been evidenced by Scanning Electron Microscopy as shown in Fig. 4. The gels have a typical microstructure with chambers about 100 microns and pores of about 10 microns. Their microstructure differ from those observed on chitosan gels processed by simple neutralization wherein a lamellar architecture was present.

Another important characteristic is related to the injectability and *in vivo* gelation of chitosan/ $\beta$ -glycerophosphate solutions. Injections and gels are typically shown on Figs. 5A and 5B. Black arrow in Fig. 5A shows the gel formed *in situ* at the knee joint intraarticular zone between the collateral ligament and the patellar tendon. The other gel is formed sub-cutaneously between the skin and leg muscles. Black arrow in Fig. 5B shows the gels formed subcutaneously *in situ* at the torso region.

In chitosan/organo-phosphate gels, organo-phosphate anions contribute to the cross-linking of chitosan macromolecule chains, but not in the same way as the pure ionic cross-linking that takes place during the gelation of chitosan by inorganic divalent anions, such as sulfate, oxalate, phosphate or polyphosphate (pyrophosphates, metaphosphates or tripolyphosphates). A chitosan aqueous solution turns into gel instantaneously in presence of inorganic divalent anions and independently of the solution pH value. Furthermore, the elevation of temperature constitutes an unfavorable factor for the gelation of this kind of systems. In contrast, the gelation of chitosan/organo-phosphate solution depends on both, the final pH of chito-

san/organo-phosphate solution and the temperature. Every solution of chitosan/organo-phosphate can not be gelled, at any temperature, as long as its pH remains below 6.45, and every solution of chitosan/organo-phosphate with pH above 6.45 can be prepared at 20°C, without immediate gellation and can be stored for long time at 4°C without turning to gel. At 37°C only the chitosan/organo-phosphate solutions with pH above 6.9 can be gelled more or less rapidly. It is expected that the presence of organo-phosphate molecules in chitosan solutions directly affects electrostatic interactions, hydrophobic interactions and hydrogen bonds of chitosan chains. Thus, the main interactions involved in the formation of chitosan/organo-phosphate gels become essentially: 1) chitosan/chitosan interchain hydrogen bonding (CHITOSAN-CHITOSAN); 2) chitosan/organo-phosphate electrostatic attractions between the ammonium groups of macromolecule chains and the phosphate group of organo-phosphate molecules (CHITOSAN-PHOSPHATE); 3) chitosan-chitosan hydrophobic interactions induced through the structuring action of the polyol or sugar parts on water molecules. The structuring action of the polyol parts on water reduces the chitosan-water interactions and therefore enhances the chitosan-chitosan interactions. The nontrivial aspect of such a gelation originates essentially from the later polyol-water induced chitosan hydrophobic attractions which are enhanced upon increasing temperature (temperature-controlled gelation). At low temperatures, chitosan-water strong interactions protect the hydrated chitosan macromolecules against aggregations. Removal upon heating of the sheath of water molecules favors and strengthens chitosan-chitosan interactions, and hence induces the macromolecules association. However, the gelation would never occur if the two first attractions

(CHITOSAN-CHITOSAN & CHITOSAN-PHOSPHATE) are fully unoperational within the chitosan/organo-phosphate solution. This explains the pH-dependence that still governs the temperature-controlled gelation of chitosan/organo-phosphate systems. Although such CHITOSAN-PHOSPHATE electrostatic attractions are present, the phosphate groups can not be the unique cross-linker agent of chitosan chains due to non-compatible stearic hindrance. This significantly differentiate this gelation mechanism from the pure ionic gelation of chitosan by phosphates or polyphosphates divalent anions. A pure ionic cross-linking would not be temperature-controlled or stimulated.

This type of temperature-controlled pH-dependant gelation is specifically induced by organic mono-phosphate dibasic salt in chitosan solution, however it may be induced as well by other organic salts such as mono-sulfate salts of polyols or sugars, such as polyol-sulfate or sugar-sulfate, or mono-carboxylic acid salts of polyols or sugars. For example, in accordance with the present invention, a chitosan/glucose-1-sulfate solution is expected to gelate so as a chitosan/glucose-1-phosphate solution does.

It is also an aim of the present invention to provide an aqueous chitosan/organo-phosphate solution which can be formed and stored at low temperature (4°C) and transformed at physiological temperatures into three-dimensional stable chitosan/organo-phosphate gel. It includes nontoxic biocompatible components for mammalian or human environments with both components and processes having low toxicity effects towards living biologicals and preserving the cellular viability. The gel also provides good mechanical/ handling performances for long periods of time at the physiological temperature and in physiological aqueous media contain-

ing amino-acid, ions and proteins. Chitosan derivatives may be selected as well as to process chitosan/organo-phosphate gels, and comprise N,O-substituents of chitosan.

5           The expression "organo-phosphates (salt)" refers herein, without limitation, to mono-phosphate dibasic salts of polyols or sugars, such as polyol-phosphate dibasic salts or sugar-phosphate dibasic salts. Organo-sulfates (salt) also refer herein to mono-sulfate salts  
10 of polyols or sugars, such as polyol-sulfate salts or sugar-sulfate salts. The preferred organo-phosphate salts may be selected from mono-phosphate dibasic salts of glycerol, including glycerol-2-phosphate, sn-glycerol 3-phosphate and 1-glycerol-3-phosphate salts  
15 (alpha-glycerophosphate or beta-glycerophosphate), mono-phosphate dibasic salts of histidinol, acetol, diethylstilbestrol, indoleglycerol, sorbitol, ribitol, xylitol, arabinitol, erythritol, inositol, mannitol, glucitol, palmitoyl-glycerol, linoleoyl-glycerol,  
20 oleoyl-glycerol or arachidonoyl-glycerol, and mono-phosphate dibasic salts of fructose, galactose, ribose, glucose, xylose, rhamnulose, sorbose, erythrulose, deoxy-ribose, ketose, mannose, arabinose, fuculose, fructopyranose, ketoglucose, sedoheptulose, trehalose,  
25 tagatose, sucrose, allose, threose, xylulose, hexose, methylthio-ribose or methylthio-deoxy-ribulose. Other mono-salts of interest (sulfate, carboxylate) may be derived from the same polyols or sugars.

          The expression "glycerophosphate or glycerophosphate"  
30           refers herein to both alpha-glycerophosphate or beta-glycerophosphate isomers. Alpha-glycerophosphate is undistinctively referred for glycerol-3-phosphate (all optical enantiomers) while beta-glycerophosphate is similarly referred for glycerol-2-phosphate.

35           The expression "three-dimensional" refers herein

to the fact that the polymeric solution is simultaneously gelated and shaped by the mold wherein the solution was initially poured. Gels can be produced in glass or plastic bechers, dishes, tubes or between two plates so as to obtain any expected shapes.

The expression "in situ gelation" refers herein to the formation of chitosan/organo-phosphate gels by injecting the liquid chitosan/glycerophosphate solution within specific sites of mammalian or human environments, e.g. any tissues (muscles, bone, ligaments, cartilages) and organs. Gelation *in situ* allows complete and precise filling of tissue defects or body cavities. The gelation of the chitosan/organo-phosphate mixture is induced by the physiological temperature.

The expression "endothermal gelation" refers herein to the thermal mechanism of the chitosan/organo-phosphate solution which enables the solution to gelate upon standing at the desired temperature. Induction of sol to gel transitions of chitosan/organo-phosphate systems requires energy via, for example, the temperature.

The expression "cells or cellular matters" refers herein to living biologicals, such as isolated cells, cellular dispersion, cell aggregates, cell spheroids or cells adhered to solid microspheres particles, that are encapsulated within the chitosan/organo-phosphate gels.

The expression "in situ forming" refers herein to the procedure of administrating the ungelated chitosan/organo-phosphate liquid solution to a body site (e.g. connective tissues, body conduits, articular cavities, fractures, bone defects...), and inducing and ensuring within the body site at the physiological temperature a complete gelation of the polysaccharide solution into a gel.

### Formation of chitosan/organo-phosphate gels

The selected organo-phosphate salt was herein glycerophosphate, but similar results were reached with  
5 other mono-phosphate dibasic salts of polyols or Sugars. Chitosan in powder form is dissolved in an aqueous acidic solution until the occurrence of a clear solution is obtained. The proportion of chitosan varies from 0.5 to 5.0% w/v, preferentially from 1.0 to 3.0%  
10 w/v. The pH of the aqueous chitosan solution ranges from 4.5 to 5.5. Aqueous chitosan solutions can be sterilized either by filtration with in-line sterile filters (0.22 micrometer) or by steam-autoclaving (120°C). Sterilization of the chitosan/glycerophosphate  
15 gels can not be filtered due to the viscosity or steam-autoclaved due to the thermal sensitivity, but can be performed by gamma-irradiation or reached through strictly sterile procedures. Freshly-prepared aqueous chitosan solutions are stored preferably at low positive temperature (4°C). Glycerophosphate  
20 felt in fine powder form is added to, and dissolved within, the aqueous chitosan solution at a temperatures ranging from 4 to 15°C, preferentially 10°C. When a clear homogeneous chitosan/glycerophosphate aqueous solution with  
25 a pH ranging from 6.5 to 7.2 is attained, the said solution is poured into the desired receiver, and hold to appropriate temperature to gel. Glycerophosphate felt in form of aqueous solution may be also used.

Depending on their final pH, the chito-  
30 san/glycerophosphate solutions are expected to lead either to thermally reversible or irreversible gel. Reversible gels arise from chitosan/glycerophosphate solutions having a pH comprising between 6.5 and 6.9, while the irreversible gels originate from chito-  
35 san/glycerophosphate solutions having a pH above 6.9.



The nature of the acid that are used for the acidic chitosan solutions does not influence fundamentally the sol to gel transition of the chitosan/glycerophosphate system. The final pH within a chitosan/glycerophosphate solution is dependent upon the pH of the water/acid solution as well as the chitosan and glycerophosphate concentrations. As chitosan and glycerophosphate are two alkaline components, they tend to increase the pH of the acidic solution wherein they are dissolved. Concentrations in chitosan and glycerophosphate can be balanced to reach the appropriate pH of the chitosan/glycerophosphate solution, while taking into consideration the solubility limit of both components, and particularly the one of chitosan.

**15 Three-dimensional monolithic gels**

The selected organo-phosphate salt was herein glycerophosphate, but similar results were reached with other mono-phosphate dibasic salts, monosulfate salts or monocarboxylate salts of polyols or sugars. The receiver or mold filled with chitosan/glycerophosphate solution are heated at a temperature ranging from 30 to 60°C, preferentially 37°C. The gelation of chitosan/glycerophosphate solution at 37°C can be performed within a common cell culture incubator. The solution is maintained at the desired temperature until it turns into a gel after a period which ranges from some days to a week (at 30°C) to few minutes (at 60°C). At 37°C, the gelation of chitosan/glycerophosphate solution occurs in 1 hour approximately. Once a three-dimensional chitosan/glycerophosphate gel is formed, the said gel is unmolded and washed in distilled water. Chitosan/glycerophosphate gels remain stable and keep their three-dimensional shape even at high temperature, 120°C (in autoclave).

### *In situ* formation of gels

The selected organo-phosphate salt was herein glycerophosphate, but similar results were reached with other mono-phosphate dibasic salts, monosulfate salts or monocarboxylate salts of polyols or sugars. *In situ* gelation of the chitosan/glycerophosphate solution can be conducted by dispensing the solution from a hypodermic syringe. If needed, the solution may be pre-gelated (initiate the thermal gelation) by keeping the syringe and chitosan/glycerophosphate solution at desired temperature, ideally 37°C, until the first signs of gelation appear. The ready-to-gel chitosan/glycerophosphate mixture is then administrated so as to fill tissue defects or cavities and complete *in situ* the gelation process (at 37°C). Injection of chitosan/glycerophosphate solutions is however limited by the viscosity of the solutions which controls the injectability or extrudability of the solutions. A needle having a gauge of 20 and below are ideal materials for injection of such gel solution. Body cavities and tissue defects act as recipients for the solution, but the liquid materials remain in an open aqueous environment. The conformability and diffusability of the chitosan/glycerophosphate solutions is dependent upon the solution and material properties. Increased viscosity results in formation *in situ* of more compact and less conformable gels.

### Encapsulation of living biologicals with chitosan/glycerophosphate gels

The selected organo-phosphate salt was herein glycerophosphate, but similar results were reached with other mono-phosphate dibasic salts, monosulfate salts or monocarboxylate salts of polyols or sugars. Living cells or cellular matters were prepared using current cell culture techniques. Cells or cellular matters were incorporated and homogenized at low positive tempera-

tures, ranging from 4 to 20°C, ideally 20°C, into the aqueous chitosan/glycerophosphate solution. The cells or cellular matters loaded with chitosan/glycerophosphate mixtures were poured in the  
5 desired dishes or wells and incubated at 37°C. Minimal or supplemented cell culture medium was added to the dishes or wells containing the cells or cellular matters loaded with chitosan/glycerophosphate materials so as to maintain alive and metabolically active the living  
10 encapsulated biologicals. Cell culture medium was renewed every 2 days following the formation of the chitosan/glycerophosphate gels.

The viability of cells within the solution and gel is potentially reduced by abnormal osmolarity. Chitosan/glycerophosphate systems have changing osmolarities depending upon the ionic glycerophosphate component. The highest is the glycerophosphate salts content, the highest is the osmolarity of the solution, and the greatest is the impairment for the cell viability. Ideal osmolarity for bioencapsulation would be  
20 around 270 to 340 mOsmol/kg. Injection and *in situ* gelation of chitosan/glycerophosphate materials loaded with living cells or cellular matters can be assessed in a similar way. Cells or cellular matters are introduced at a low positive temperature within the aqueous  
25 chitosan/glycerophosphate solutions prior to injection and gelation. There exist a direct relationship between the glycerol-2-phosphate disodium salt content and the osmolarity. To reduce such osmolarity problems, the final pH of the chitosan/glycerophosphate can be  
30 adjusted to its desired value while keeping the glycerophosphate content as low as possible. However, a low glycerophosphate content limit must be reached for processing the temperature-controlled pH-dependant  
35 gelation.

#### Therapeutic use and other uses of chitosan-based Gels

A chitosan/organo-phosphate or organo-sulfate gel as previously described is an ideal material for drug delivery system. Such a *in situ* gel-like forming vehicle, wherein a solid particulate or water-soluble additive is incorporated prior to the gelation, can be administered topically, directly to the body site to be treated or diagnosed. Anti-bacterial, anti-fungal, steroidal or non-steroidal anti-inflammatory, anti-cancer, anti-fibrosis, anti-viral, anti-glaucoma, miotic and anti-cholinergics, anti-psychotic, anti-histaminic and decongestant, anesthetic and anti-parasitic agents may be incorporated within the composition and gel. In a similar fashion, polypeptides or non-living pharmaceutical agents may be incorporated within the composition or gel for restorative, reconstructive or regenerative purposes.

Living microorganisms, plant cells, animal cells or human cells may be entrapped identically within the polysaccharide gel by introduction prior to the gelation. The cells or micro-organisms loaded gels may be applied to biotechnological purposes in medicine or in other industrial areas. Chitosan-based *in situ* forming gels can be formed sub-cutaneously, intramuscularly, intra-peritoneally or within biological connective tissues, bone defects, fractures, articular cavities, body conduits or cavities, eye cul-de-sac, solid tumor vasculatures, etc...

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

## Typical gelation of a chitosan/organo-phosphate system

## Experiment 1:

Typical experiment was carried out by dissolving  
5 0.2 g of chitosan in 10 ml of aqueous acetic acid solution (0.1M). The pH of the acetic acid solution has been beforehand adjusted to 4.0 by adding droplets of potassium hydroxide solution (1M). The 2% (w/v) chitosan solution so obtained had a pH of about 5.6. Then,  
10 0.800 g of glycerophosphate disodium salt pentahydrate were added to and dissolved in the chitosan solution at 10°C. The pH of the resulting homogeneous liquid mixture become 7. This mixture was disposed in a glass scintillation vial in the incubator at 37°C for 2 hours, enough  
15 time to achieve bulk-gelation process. The resulting bulk gel was immersed in renewed baths of distilled water in order to remove the excess of glycerophosphate salt.

A similar result was reached when the glycerophosphate disodium salt (or glycerol-2-phosphate disodium salt) was replaced by the alpha-glycerophosphate disodium salt (or glycerol-3-phosphate disodium salt).

## Experiment 2:

A homogenized chitosan/glycerophosphate solution  
25 was prepared as in Experiment 1 and disposed in a dual gel caster having a glass plates gel sandwich with a 1.6 mm interspaces, and the system was kept in an oven at 37°C. The formation of a gel membrane was reached within 2 hours and the membrane was unmolded from the  
30 gel caster.

## Experiment 3:

A 0.110 g of fumed silica under solid particle form (AEROSIL) was dispersed within a solution prepared by dissolving 0.200 g of chitosan in 10 ml of aqueous  
35 acetic acid solution. . A 0.800 g of glycerophosphate

disodium salt pentahydrate was added to the chitosan-silica dispersion. The resulting composition was disposed in a glass scintillation vial in water bath kept at 37°C. The gelation of the chitosan/glycerophosphate component was observed within 2 hours, and the chitosan/glycerophosphate gel includes dispersed solid silica particles.

#### Experiment 4:

A 0.200 g of chitosan was dissolved in acetic acid solution as in Experiment 1. A 1.239 g of glucose-1-phosphate disodium salt tetrahydrate was added and dissolved so as to reach a clear chitosan/glucose-1-phosphate solution. This chitosan/glucose-1-phosphate solution placed in a glass scintillation vial was maintained at 37°C. The Sol to Gel transition occurs at 37°C within 3 hours. The resulting bulk gel was immersed in renewed baths of distilled water in order to remove the excess of glucose-phosphate salt.

The experiment was conducted as described in Experiment 4 except that the 1.239 g of glucose-1-phosphate salt was replaced by 0.100 g of fructose-6-phosphate disodium salt dihydrate.

#### EXAMPLE II

##### Effect of composition on pH of solution and occurrence of gelation

A mother acidic solution made of a Water/Acetic acid was prepared for all experiments. The pH of this mother acidic solution was adjusted to 4.0. High molecular weight (M.w. 2,000,000) Chitosan powder was added and dissolved in a volume of the mother acidic solution so as to produce Chitosan solutions having Chitosan proportions ranging from 0.5 to 2.0% w/v (Table 1). Table 1 reports the measured pH for the different samples.

Table 1

## Chitosan Aqueous Solutions and pH levels

Chitosan conc. (w/v)	0.5	1.0	1.5	2.0
pH of Chitosan Sol.	4.68	4.73	5.14	5.61

Glycerophosphate was added to the chitosan solutions and induces a pH increase. Table 2 shows the effect of glycerophosphate concentration on different chitosan solution. The concentration of glycerophosphate ranges from 0.065 to 0.300 mol/L. The chitosan/glycerophosphate solutions in glass vials were maintained at 60 and 37°C, and bulk and uniform gelation was noted within 30 minutes at 60°C and 6 hours at 37°C (Table 2 and Fig. 1). Chitosan and beta-glycerophosphate components individually influence the pH increase within the aqueous solutions, and consequently influence the Sol to Gel transition. As well as the dissolved materials, the initial pH of the mother water/acetic acid solution would also influence the Sol to Gel transition, but this potential effect seems to be limited by the counter-action of the chitosan solubility which depends on the pH of the solution.

Table 2

## Gelation of Chitosan/Glycerophosphate Compositions

Chitosan conc. (w/v)	1.5			2.0		
pH of Chitosan Sol.	5.14			5.61		
GP conc. (mol/L)	0.130	0.196	0.260	0.130	0.196	0.260
pH of Chitosan-GP Sol.	6.64	6.83	6.89	6.78	6.97	7.05
Gelation						
60°C	< 30 min.	< 30 min.	< 30 min.	< 30 min.	< 30 min.	< 30 min.
37°C	No	No	No	No	< 6 hrs	< 6 hrs

EXAMPLE III

## Thermal reversibility of gelation of chitosan/glycerophosphate systems

A first chitosan/glycerophosphate solution was prepared from a 10 ml of mother water/acetic acid solution of pH 4.0 by adding and dissolving 0.200 g of chitosan and 0.800 g of glycerophosphate disodium salt

pentahydrate. The resulting chitosan/glycerophosphate solution has a pH about 7.05 and is heated at 60°C where it gels rapidly. The chitosan/glycerophosphate gel is cooled at a temperature about 0-4°C, but no transition is observed with time and the gel remains stable at 4°C. This chitosan/glycerophosphate gel is thermo-irreversible.

A second chitosan/glycerophosphate solution was prepared from a 10 ml of mother water/acetic acid solution of pH 4.0 by adding and dissolving 0.100 g of chitosan and 0.800 g of glycerophosphate disodium salt pentahydrate. The chitosan/glycerophosphate solution has a pH about 6.78 and is heated at 60°C for gelation. The resulting chitosan/glycerophosphate gel is then cooled at a temperature about 0-4°C, and a Gel to Sol transition is observed after a period of time. The gel returns to a liquid solution at low positive temperatures, for instance at 4°C. When this solution is reheated at 60°C, the reverse mechanism (Sol to Gel transition) appears again and a gel re-forms. The Sol to Gel to Sol transition is reproducible between temperatures at 4 and 60°C. This chitosan/glycerophosphate gel is thermo-reversible. The thermo-reversibility of the Sol to Gel transition of the chitosan/glycerophosphate systems is found to be predominantly dependant on the pH of the chitosan/glycerophosphate solution. An example of pH and observed reversibility is give in Table 3. Experimental observations on chitosan/glycerophosphate systems have shown that thermal reversibility of the Sol to Gel mechanism changes at a pH of the chitosan/glycerophosphate solutions in the vicinity of 6.9. A reversible gelation of chitosan/glycerophosphate solutions occurs when the pH is comprised between 6.5 and 6.9. A very similar result was observed with other



mono-phosphate dibasic salts of polyols or sugars such as glucose-1-phosphate salts.

**Table 3**

**Effect of pH on Sol to Gel Reversibility for a 2.0% w/v Chitosan Concentration**

5

GP conc. (mole/L)	0.130	0.163	0.196	0.260
pH of Chitosan-GP Sol.	6.78	6.87	6.97	7.05
Reversibility of Sol to Gel 4°C - 60°C	Reversible, Cycle	Reversible, Cycle	Irreversible	Irreversible

**EXAMPLE IV**

***In situ* Gelation of chitosan/glycerophosphate materials**

Adult New-Zealand White rabbits were anesthe-  
tized by intra-venous administration of Sodium Pen-  
tosorbital (1 ml/kg) and maintained under anesthesia  
for 3 hours. After 3 hours, the animals were sacrificed  
by anesthetic overdose and the experiments were contin-  
ued post-mortem. The animal was maintained on the back,  
and the localized zones of the upper limbs and torso  
region were shaved. The skin was incised so as to free  
the sub-cutaneous fibrous membranes and muscle of the  
limb.

Two chitosan/glycerophosphate solutions were  
prepared previously, disposed in hypodermic syringes  
and maintained at a low temperature (4-10°C): the solu-  
tion I was a 2.7 % w/v low M.w. chitosan and 9.0 % w/v  
glycerophosphate while the solution II was a 2.5%  
medium M.w. chitosan and 9.0% w/v glycerophosphate.  
Syringes were equipped with No. 21 gauge needles. The  
chitosan/glycerophosphate solutions were not prepared  
or kept under strict sterile conditions since the ani-  
mal experiments were performed within a period of time  
about 4-5 hours. A volume (a) of 1.0-1.5 ml of the  
solution I was injected sub-cutaneously within the  
fibrous membranes while a second volume (b) of 2.0 ml  
of the solution I was injected through the knee-joint

capsule into the articular cavity. A volume (c) of 1.0 ml of the solution II was injected sub-cutaneously at the torso region. All injection sites were recovered with the excised tissues where necessary. The gelation *in situ* was allowed for a period of 3 hours, then the sites were re-opened or excised and the *in situ* formed gels were collected.

Table 4  
Injections and formation *in situ* of Gels

	[chitosan] % w/v	Volume	Remarks
Subcutaneous, Legs.	2.7	1.5	Gel expands and conforms to the tissue surface.
Intraarticular, Knee joint	2.7	2.0	Gel expands and conforms to the joint volume contour.
Subcutaneous, Torso	2.5	1.0	Gel does not expand and retains its drop shape.

10

Example V

**Encapsulation of mammalian cells**

A 2.5% w/v chitosan aqueous solution was prepared as previously described. A 1.98 g of the chitosan solution was admixed with 0.18 g of beta-glycerophosphate disodium salt, 0.4 ml of Dubelcco Modified Eagle Medium F12 wherein animal chondrocytes are dispersed, and 0.2 ml of Dubelcco Modified Eagle Medium F12. Chondrocytes were isolated from calf shoulder cartilage surfaces and collected from primary monolayer cultures. Dubelcco Modified Eagle Medium F12 comprises dexamethasone, ascorbic acid and 10% fetal bovine serum. All Chitosan solutions, chitosan/glycerol-2-phosphate solutions and procedures were sterile. Once the chondrocytes in chitosan/glycerol-2-phosphate dispersion is casted, it is placed in an incubator at 37°C until the gelation. Gelation of chitosan/glycerol-2-phosphate system is observed within 2 hours. Viability tests on such chondrocytes-loaded chitosan/glycerol-2-phosphate

gels indicate a range from 10% to 70% of alive chondrocytes. Encapsulation of micro-organisms, plant cells, animal cells or human cells within chitosan/glycerol-2-phosphate gels can be performed with changing properties depending the expected viability.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A polysaccharide based gel which comprises:
  - a) 0.1 to 5.0% by weight of chitosan or a chitosan derivative; and
  - b) 1.0 to 20% by weight of a salt of polyol or sugar selected from the group consisting of mono-phosphate dibasic salt, mono-sulfate salt and a mono-carboxylic acid salt of polyol or sugar;wherein said gel is induced and stable within a temperature range from 20 to 70°C and is adapted to be formed and/or gelated *in situ* within a tissue, organ or cavities of an animal or a human.
2. A gel according to Claim 1, wherein said salt is a mono-phosphate dibasic salt selected from the group consisting of glycerol, comprising glycerol-2-phosphate, sn-glycerol 3-phosphate and L-glycerol-3-phosphate salts.
3. A gel according to Claim 1, wherein said salt is a mono-phosphate dibasic salt and said polyol is selected from the group consisting of histidinol, acetol, diethylstilbestrol, indole-glycerol, sorbitol, ribitol, xylitol, arabinitol, erythritol, inositol, mannitol, glucitol and a mixture thereof.
4. A gel according to Claim 1, wherein said salt is a mono-phosphate dibasic salt and said sugar is selected from the group consisting of fructose, galactose, ribose, glucose, xylose, rhamnulose, sorbose, erythrulose, deoxy-ribose, ketose, mannose, arabinose, fuculose, fructopyranose, ketoglucose, sedoheptulose, trehalose, tagatose, sucrose, allose, threose, xylu-

lose, hexose, methylthio-ribose, methylthio-deoxy-ribose, and a mixture thereof.

5. A gel according to Claim 1, wherein said salt is a mono-phosphate dibasic salt and said polyol is selected from the group consisting of palmitoyl-glycerol, linoleoyl-glycerol, oleoyl-glycerol, arachidonoyl-glycerol, and a mixture thereof.

6. A gel according to Claim 1, wherein said gel composition is selected from the group consisting of chitosan- $\beta$ -glycerophosphate, chitosan- $\alpha$ -glycerophosphate, chitosan-glucose-1-glycerophosphate, and chitosan-fructose-6-glycerophosphate.

7. A gel according to Claim 1, wherein solid particulate or water-soluble additives are incorporated within said polysaccharide gel prior to the gelation.

8. A gel according to Claim 1, wherein drugs, polypeptides or non-living pharmaceutical agents are incorporated within said polysaccharide gel prior to the gelation.

9. A gel according to Claim 1, wherein living microorganisms, plant cells, animal cells or human cells are encapsulated within said polysaccharide gel prior to the gelation.

10. A gel according to Claim 1, wherein said gel is formed *in situ* sub-cutaneously, intra-peritoneally, intra-muscularly or within biological connective tissues, bone defects, fractures, articular cavities, body conduits or cavities, eye cul-de-sac, or solid tumors.

11. A gel according to Claim 10, wherein said gel is a carrier for delivering pharmaceutical agents *in situ*.

12. A method for producing a polysaccharide gel solution according to Claim 1, which comprises the steps of:

- a) dissolving a chitosan or a chitosan derivative within an aqueous acidic solution of a pH from about 2.0 to about 5.0 to obtain an aqueous polysaccharide composition having a concentration of 0.1 to 5.0% by weight of a chitosan or of a chitosan derivative;
- b) dissolving 1.0 to 20% by weight of a salt of polyol or sugar, wherein said salt is selected from the group consisting of mono-phosphate dibasic salt, mono-sulfate salt and a mono-carboxylic acid salt, in said aqueous polysaccharide composition of step a) to obtain a polysaccharide gel solution, wherein said polysaccharide gel has a concentration of 0.1 to 5.0% by weight of a chitosan or a chitosan derivative, and a concentration of 1.0 to 20% by weight of a salt of a polyol or sugar, and has a pH from about 6.4 to about 7.4.

13. The method of Claim 12, which further comprises a step c) after step b),

- c) heating said polysaccharide gel solution at a solidifying temperature ranging from about 20°C to about 80°C until formation of a polysaccharide gel.

14. The method of Claim 12 or 13, wherein a pharmaceutical agent is added to the polysaccharide gel solution of step b).

15. The method of Claim 13 or 14, which further comprises a step i) after step b),

- i) dispensing for geletion the polysaccharide gel solution into a desired receiver, either in a mold or within a tissue, organ or body cavity.

16. The method of Claim 12, 13 or 14, wherein said aqueous acidic solution is prepared from organic or inorganic acids selected from the group consisting of acetic acid, ascorbic acid, salicylic acid, phosphoric acid, hydrochloric acid, propionic acid, formic acid, and a mixture thereof.

17. The method of Claim 12, 13 or 14, wherein said salt is a mono-phosphate dibasic salt of glycerol, wherein said glycerol is selected from the group consisting of glycerol-2-phosphate, sn-glycerol 3-phosphate and L-glycerol-3-phosphate salts.

18. The method of Claim 17, wherein said glycerophosphate salt is a selected from the group consisting of glycerophosphate disodium, glycerophosphate dipotassium, glycerophosphate calcium, glycerophosphate barium and glycerophosphate strontium.

19. The method of Claim 12, 13 or 14, wherein said salt is a mono-phosphate dibasic salt of a polyol, and said polyol is selected from a group comprising histidinol, acetol, diethylstilbestrol, indoleglycerol, sorbitol, ribitol, xylitol, arabinitol, erythritol, inositol, mannitol, glucitol, and a mixture thereof.

20. The method of Claim 12, 13 or 14, wherein said salt is a mono-phosphate dibasic salt of a sugar, and

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said sugar is selected from a group comprising fructose, galactose, ribose, glucose, xylose, rhamnose, sorbose, erythrulose, deoxy-ribose, ketose, mannose, arabinose, fuculose, fructopyranose, ketoglucose, sedoheptulose, trehalose, tagatose, sucrose, allose, threose, xylulose, hexose, methylthio-ribose, methylthio-deoxy-ribulose, and a mixture thereof.

21. The method of Claim 12, 13 or 14, wherein said salt is a mono-phosphate dibasic salt of a polyol, and said polyol is selected from the group consisting of palmitoyl-glycerol, linoleoyl-glycerol, oleoyl-glycerol, arachidonoyl-glycerol, and a mixture thereof.

22. The method of Claim 12, 13 or 14, wherein said salt is a mono-phosphate dibasic salt and said phosphate is selected from the group consisting of a phosphate disodium, phosphate dipotassium, phosphate calcium, phosphate barium and phosphate strontium.

23. The method of Claim 12, wherein said polysaccharide gel solution is kept in a stable ungelled liquid form at a temperature ranging from about 0°C to about 20°C.

24. The method of Claim 13, wherein said solidifying temperature is ranging from about 37°C to about 60°C.

25. The method of Claim 24, wherein said solidifying temperature is about 37°C.

26. The method of Claim 12, 13 or 14, wherein the molecular weight of chitosan is ranging from about 10,000 to 2,000,000.



27. The method of Claim 12, 13 or 14, wherein the polysaccharide gel is thermoirreversible or thermoreversible by adjusting the polysaccharide gel pH.

28. The method of Claim 27, wherein polysaccharide gel is thermoirreversible when the pH of said polysaccharide gel solution is  $>6.9$ .

29. The method of Claim 27, wherein polysaccharide gel is thermoreversible when the pH of said polysaccharide gel solution is  $<6.9$ .

30. The method of Claim 12, 13 or 14, wherein solid particulate additives are added to the polysaccharide gel solution of step b).

31. The method of Claim 15, wherein said polysaccharide gel solution is introduced within an animal or human body by injection or endoscopic administration, and gelled *in situ* at a temperature of about  $37^{\circ}\text{C}$ .

32. Use of the polysaccharide gel of Claim 1 for producing biocompatible degradable materials used in cosmetics, pharmacology, medicine and/or surgery.

33. The use according to Claim 32, wherein the gel is incorporated as a whole, or as a component, into implantable devices or implants for repair, reconstruction and/or replacement of tissues and/or organs, either in animals or humans.

34. The use according to Claim 32, wherein the gel is used as a whole, or as a component of, implantable, transdermal or dermatological drug delivery systems.

35. The use according to in Claim 32, wherein the gel is used as a whole, or as a component of, ophthalmological implants or drug delivery systems.

36. Use of the gel according to Claim 32 for producing cells-loaded artificial matrices that are applied to the engineering and culture of bioengineered hybrid materials and tissue equivalents.

37. The use according to Claim 36, wherein the loaded cells are selected from the group consisting of chondrocytes (articular cartilage), fibrochondrocytes (meniscus), ligament fibroblasts (ligament), skin fibroblasts (skin), tenocytes (tendons), myofibroblasts (muscle), mesenchymal stem cells and keratinocytes (skin).

38. The use according to Claim 37, wherein the cells-loaded gel and derived products are devoted to the culture and engineering of artificial articular cartilage and cartilaginous tissues and organs, either for surgical or laboratory testing applications.

39. The use according to Claim 37, wherein the cells-loaded gel and derived products are devoted to the processing and engineering of living artificial substitutes for ligaments, tendons, skin, bone muscles and any metabolic organs, either for surgical or laboratory testing applications.

40. The use according to Claim 37, wherein the cells-loaded gel and derived products are applied as living substitutes for the replacement of articular cartilages, fibrocartilages, cartilaginous organs, ligaments, tendons, bone tissues or skin.

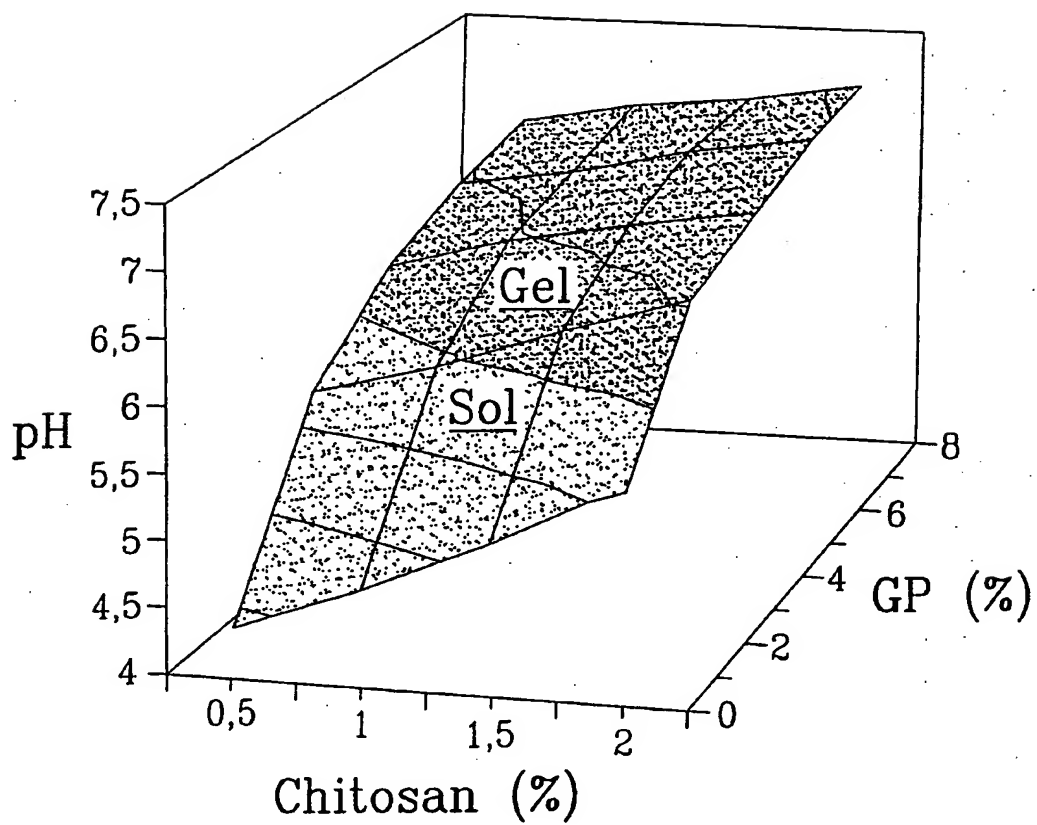
41. The use according to any one of Claim 38 to 40, wherein the said cells loaded hydrogel is gelated *in situ* to induce an ectopic formation of fibrocartilage-like or cartilage-like tissues.

42. Use of loaded polysaccharide gel as described in any of Claims 1 to 16 as injectable or implantable gel biomaterials which act as supports, carriers, reconstructive devices or substitutes for the formation *in situ* of bone-like, fibrocartilage-like or cartilage-like tissues at a physiological location of an animal or a human.

43. Use of the polysaccharide gel solution of claim 12 for producing a derived gel or hydrogel by 1) incorporating and dissolving at least one complementary polymer within said polysaccharide gel solution, and 2) by allowing said polysaccharide and complementary polymer to interact for a sufficient period of time to turn into a clear three-dimensional gel within a temperature range between 20°C to 60°C.

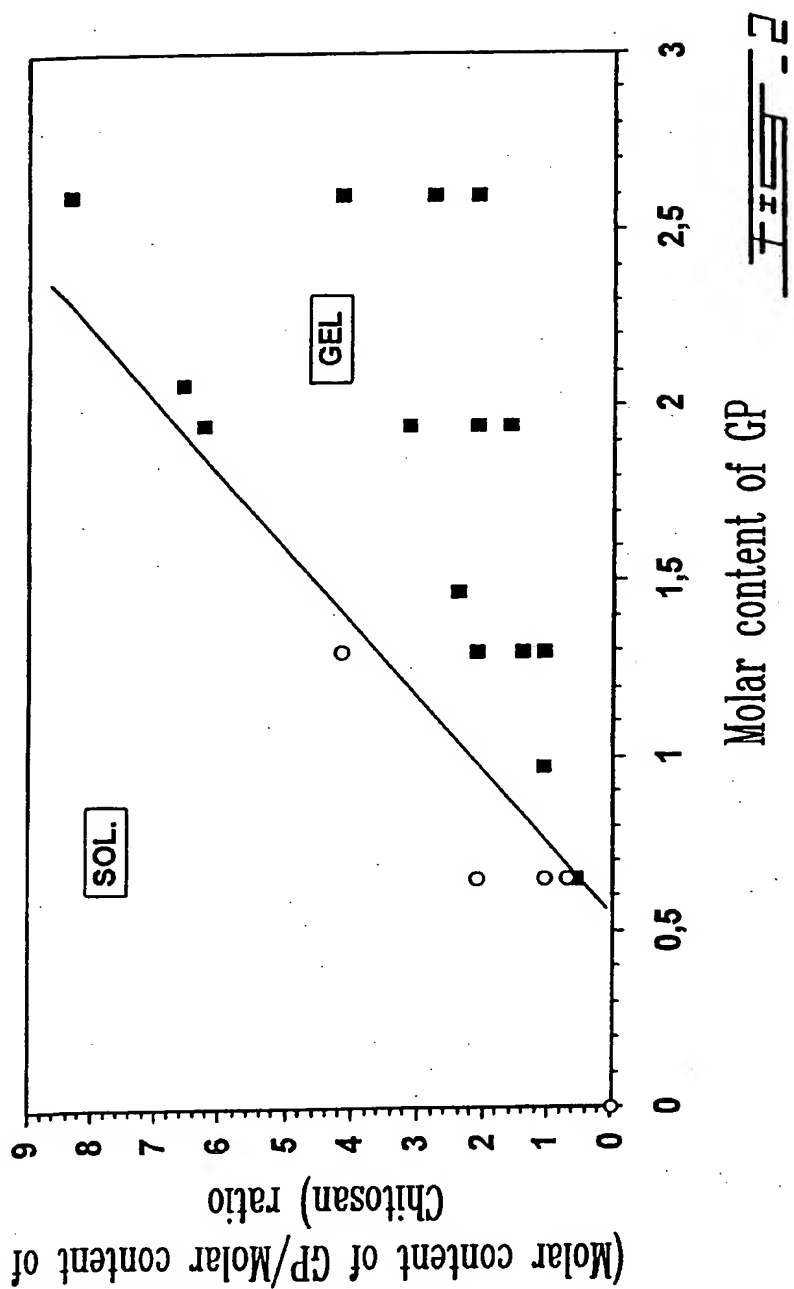
44. The use according to claim 43, wherein the complementary polymer is a non-ionic water-soluble polysaccharide or a hydroxyalkyl cellulose.

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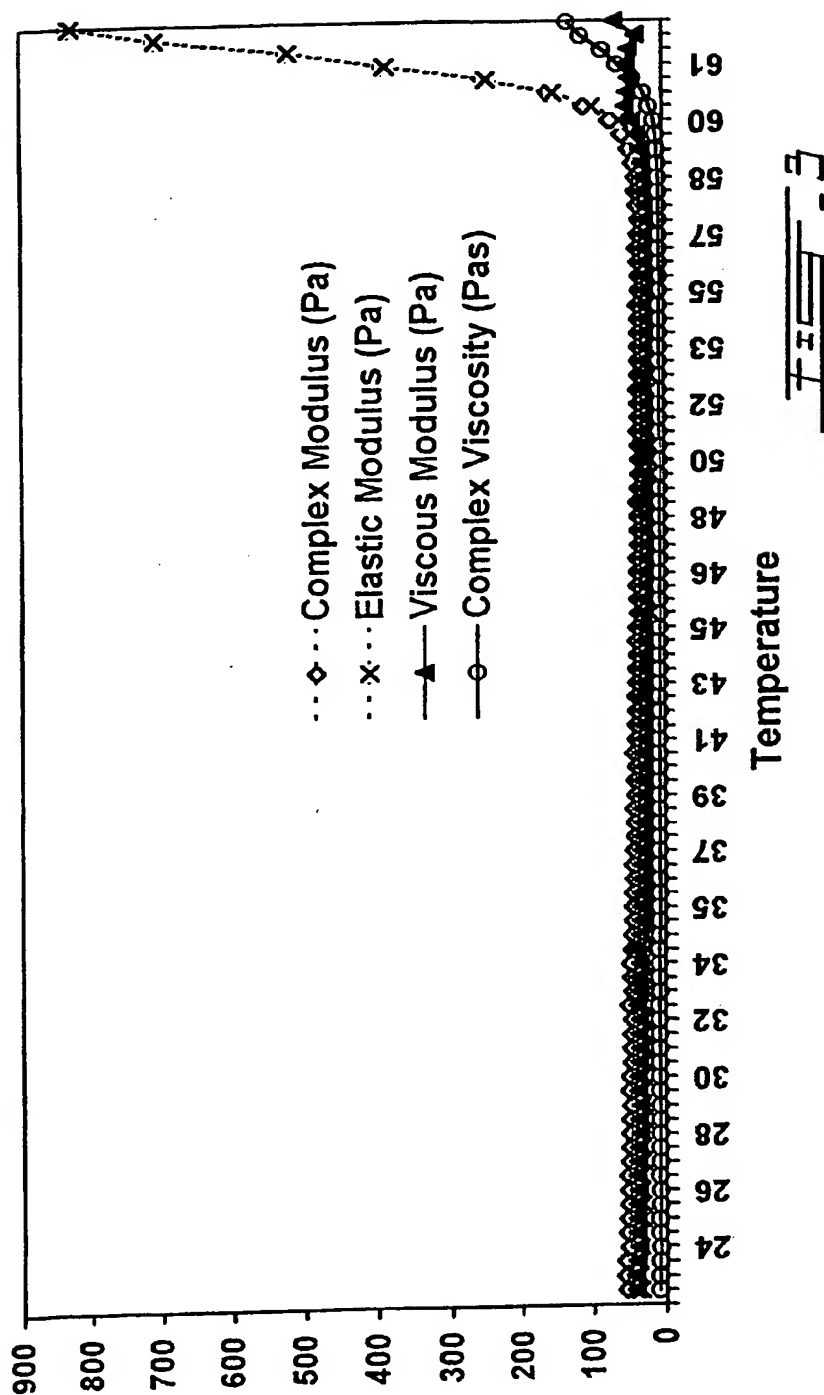
Fig. 1

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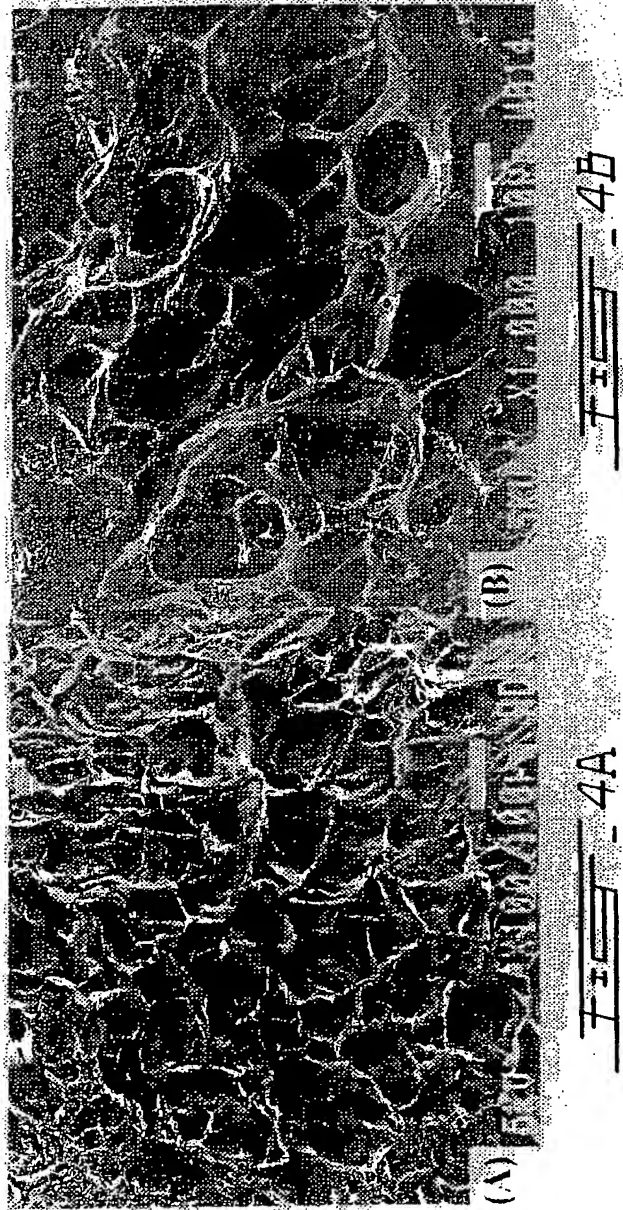


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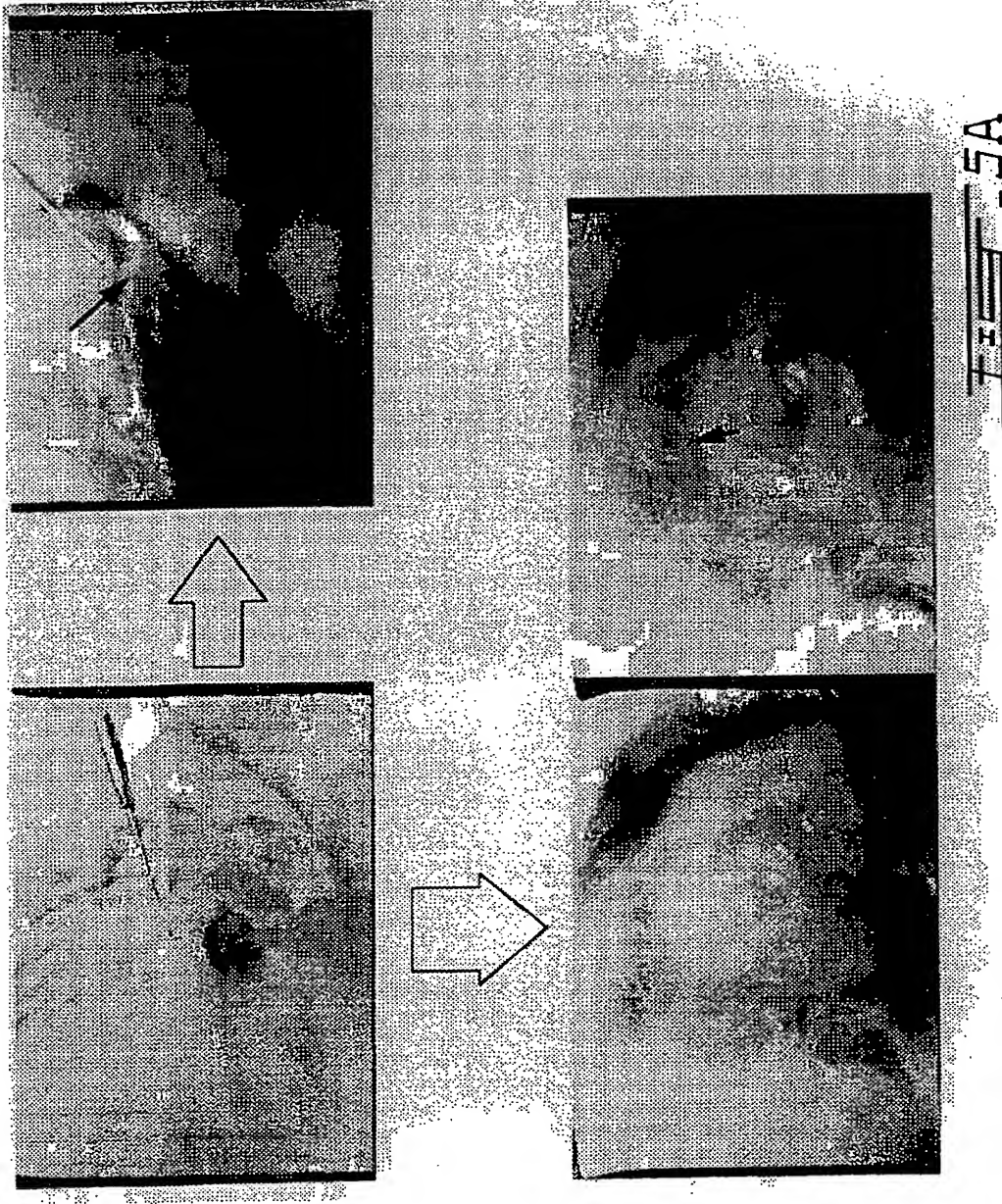
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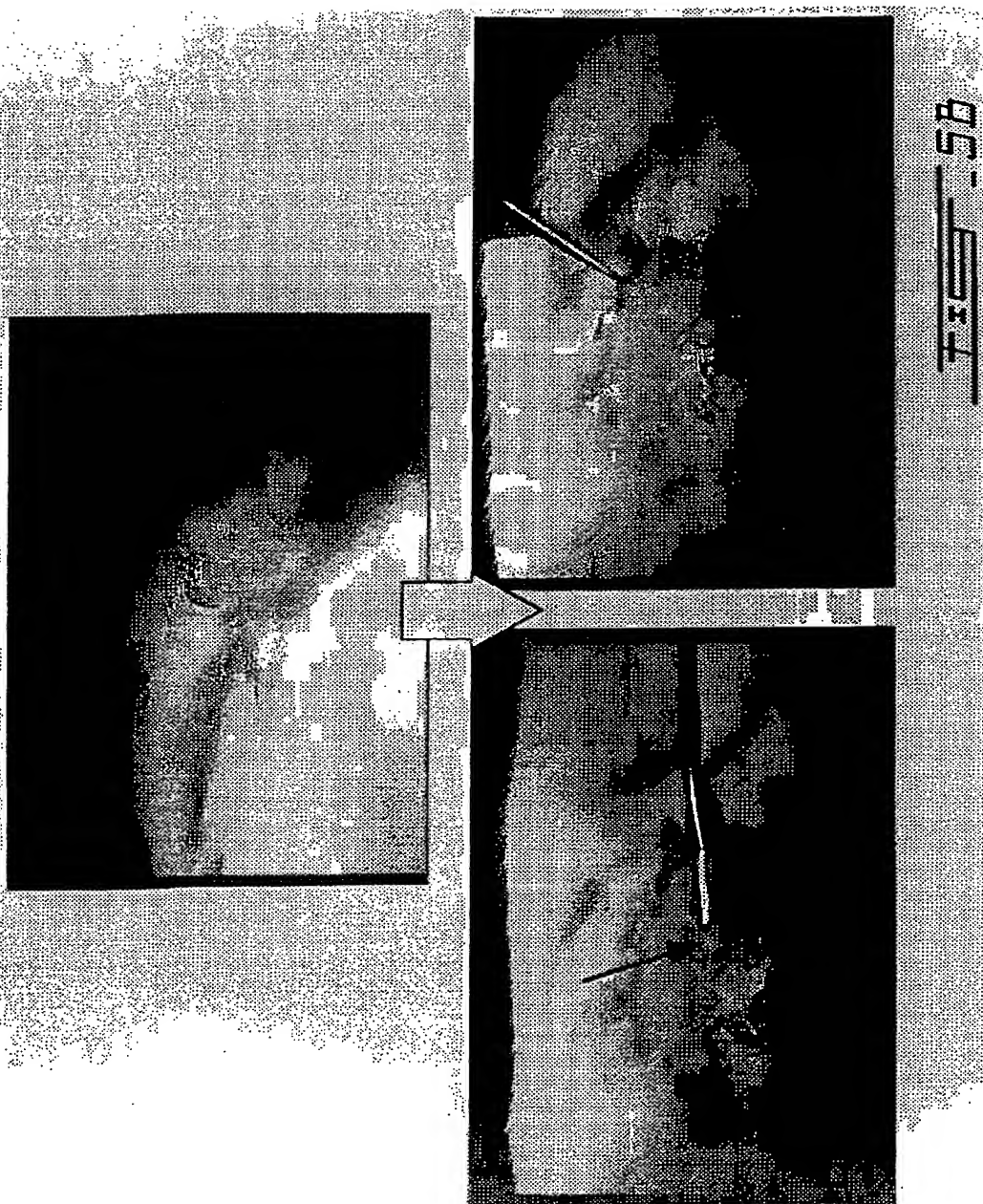
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# INTERNATIONAL SEARCH REPORT

Inter national Application No  
PCT/CA 98/00326

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61L25/00

According to International Patent Classification(IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61L C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 587 175 A (HENRY RAYMOND L ET AL) 24 December 1996 cited in the application see claims; examples ---	1-44
A	US 4 659 700 A (JACKSON DAVID S) 21 April 1987 cited in the application see claims; examples 1-3	1-44
A	US 4 647 536 A (MOSBACH KLAUS ET AL) 3 March 1987 cited in the application see claims: examples 1-9 --- -/--	1-44

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

8 October 1998

Date of mailing of the international search report

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 5 126 141 A (HENRY RAYMOND L) 30 June 1992 cited in the application see claims; examples ---	1
A	US 5 073 202 A (WALLACH DONALD F H) 17 December 1991 ---	
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